Eight antigens have been demonstrated by gel diffusion analysis of the electrophoretic bands of CB-1A and its subfractions. Highly purified preparation of the principal antigen (I) and a minor antigen (II) have been described. Explanation of the observations that chemically distinct components of CB-1A possessing different mobilities have the same antigenic specificity are discussed.

THE CHEMISTRY OF ALLERGENS. XIX.

On the Number of Antigens and the Homogeneity of the Isolated Antigens of Fraction CB-1A From Castor Beans*

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THE PURPOSE of this paper is to report on the number of antigens experimentally demonstrable in CB-1A using gel diffusion analysis and to describe the isolation of the principal antigen and a minor antigen of CB-1A in highly purified form. Although further work on CB-1A is needed, especially on the isolation of each antigen and on the correlation of antigenic and allergenic specificities, the work on castor bean allergens has been terminated for reasons not pertinent to this discussion.

CB-1A is a complex mixture of low-molecular weight proteins and polysaccharidic proteins which contains the principal allergen(s) of castor beans. CB-1A is immunologically distinct from other allergens and antigens in castor bean meal.[1-5] It was early suggested that CB-1A was a complex mixture in which many of or all of the components exhibited identical antigenic specificities.[2] In 1960, Coulson and associates[6] observed that CB-1A contained more than one antigenic specificity. Later it was demonstrated conclusively that some chemically distinct components of CB-1A had a common or identical antigenic specificity.[7,8] More recently, the major antigenic specificity of that part of CB-1A precipitated by Ponceau S was estimated as 85 per cent and the other specificities as the remaining 15 per cent.[9] Layton and associates have reported separation of CB-1A into 12 protein species by means of an ion exchange fractionation,[10] and into six or more components by paper electrophoresis.[11] They demonstrated that all of their fractions were antigenic or allergenic but they did not present conclusive experimental evidence on the number of specificities in CB-1A. The work of Layton and associates on the number of antigens in CB-1A is discussed in more detail elsewhere.[7]

Materials

Details of the preparation or refer-

*For previous paper in this series see reference No. 9.

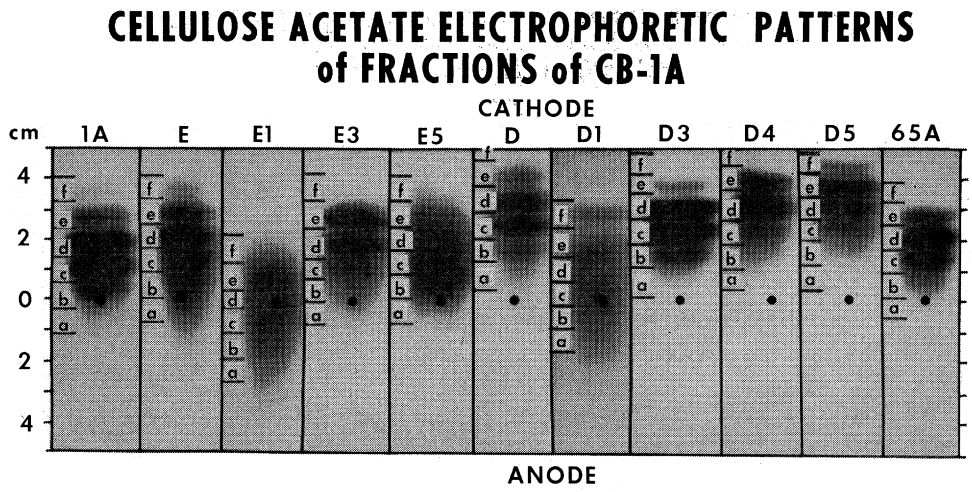

Fig. 1. Cellulose acetate electrophoretograms of CB-1A and fractions of CB-1A.

ences to original articles on the preparation of CB-1A, the ten subfractions of CB-1A and the CB-1A rabbit antiserum are given in reference 7. The cellulose acetate strips, Figure 1, are those obtained in the previously described study,[7] the strip for fraction D4 having been added. Fractions D and E were obtained on dialysis of CB-1A. The "D" series of fractions was obtained by ion exchange fractionation of the dialysate of CB-1A. The "E" series of fractions was obtained by ion exchange fractionation of that part of CB-1A remaining inside the membrane on dialysis. CB-65A is the carbohydrate-free allergenic protein obtained from CB-1A.[2]

Methods

Gel diffusion analysis of the electrophoretic bands was done as follows. Six bands, designated by the letters in Figure 1, were cut from each electrophoretic strip. Three discs, each 7 mm in diameter, were cut out with a paper punch from the portion of each band containing the CB-1A · Ponceau S complex. CB-1A rabbit antiserum, 0.15 ml, was added to the center well of an Ouchterlony plate[12] and allowed to diffuse in a moist chamber for 24 hours at 25 ± 1°. The three discs from each band were then placed in one of the six antigen wells and covered with 0.09 ml of buffered saline, pH 7.0. Thus each plate contained all of the bands from each fraction. The diffusion patterns were photographed three days after adding the discs.

Results

The results of gel diffusion analysis of CB-1A and its subfractions are shown in Figure 2. The maximum number of specificities discernible in each band, as determined by examination of the original plate, the photographic negative or the enlarged photographic print is shown in Table I. In general, examination of the negative was more critical than that of the original plate. Owing to their faintness, some lines may not be visible in the photographic reproduction, Figure 2.

The distinct antigens are identified qualitatively as follows:

Antigen I—Antigen I forms the heaviest joining line of precipitate in bands a, b, c, and d of all plates. I is

GEL DIFFUSION WITH CELL. ACETATE ELECTROPHORETIC FRACTIONS

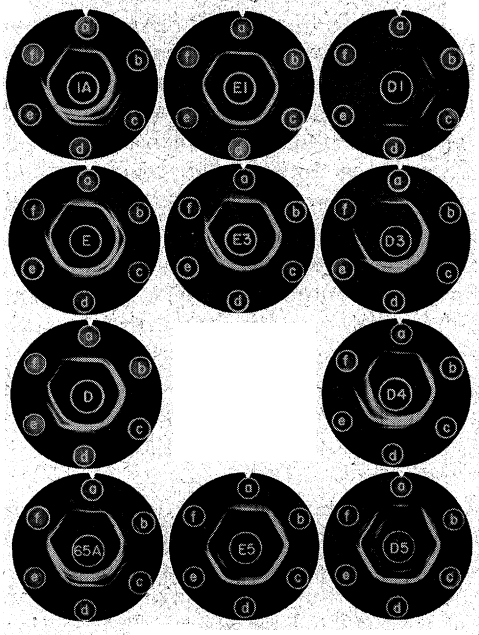

Fig. 2. Gel diffusion analysis of individual bands of the electrophoretograms of CB-1A and fractions of CB-1A. The center well contains CB-1A antiserum. The symbol in the center well designates the fraction being analyzed and the symbols in the outer wells designate the electrophoretic bands of that fraction.

TABLE I. NUMBER OF SPECIFICITIES IN THE ELECTROPHORECTIC BANDS OF CB-1A AND SUBFRACTIONS OF CB-1A AS SHOWN BY GEL DIFFUSION ANALYSIS

Fraction	Number of Specificities in Bands					
	a	b	c	d	e	f
1 A	1	4	4	4	3	2
E	2	2	4	4	3	2
E 1	2	2	3	3	3	2
E 3	2	3	2	3	3	2
E 5	1	3	3	3	4	3
D	2	3	4	4	2	2
D 1	1	2	2	2	1	1
D 3	1	3	3	4	1	1
D 4	1	2	2	5	4	2
D 5	1	2	3	3	4	4
65-A	2	3	4	4	4	2

also present in band e of most plates. Fraction E1 is almost entirely I. Antigen I is regarded as the principal antigen of CB-1A. I is identical with fraction a1.[9]

Antigen II—Antigen II forms the principal line of precipitate in band f, fraction 1A. II crosses the line of precipitate of I showing their nonidentity. II is found in all bands of fraction E and it appears to occur in all bands of fractions D, E1, E3, and 65A. In fractions E5 and D5, the principal lines of precipitate, even in band f, appear to consist of I instead of II. This relationship is not certain from the plates in Figure 2 but in Figures 10 and 11, reference 7, the principal lines of precipitate of E1 and D5 join in a reaction of identity. II is identical with fraction f2.[9]

Antigen III—Antigen III forms the line of precipitate nearest the antigen well in band e. III then becomes either the second line from the antigen wells in bands d, c, and b of fraction 1A, or continues as the line nearest the antigen wells. III appears to form the line of precipitate second from the antigen wells in bands d, c, and b of fraction 65A. III appears to be identical with the antigen forming the line of precipitate nearest the antigen well of fraction e2b.[9]

Antigen IV—Antigen IV forms the sharp, narrow line of precipitate nearest the antiserum well in bands e and d, fraction D4.

Antigen V—Antigen V forms the sharp, narrow line of precipitate in bands e and d, fraction D4 adjacent to IV.

Antigen VI—Antigen VI forms the line of precipitate in bands e and d, fraction D4 adjacent to V toward the antigen wells. Antigen VI is probably identical with: (a) that in the line of precipitate nearest the antiserum well in bands d and c, fraction 1A, as shown by the slight breaks in the inner heavy line of precipitate between bands e and

d and bands d and c, fraction 1A; (b) that in band d, fraction 65A; (c) that in band d, fraction D3; and (d) that in band e, fraction E3. The faint lines nearest the antiserum wells in E5 and D5 are probably identical with IV, V, or VI.

Antigen VII—Antigen VII forms the faint line of precipitate in band f, fraction D5 adjacent to the heavy line of precipitate in the direction of the antiserum well.

Antigen VIII—Antigen VIII forms the line of precipitate almost coinciding with the line of precipitate of antigen I, band b, fraction 1A. VIII is indicated by the precipitate breaking away from the main line of precipitate between bands b and c, fraction 1A. VIII also appears in band b, fraction D3.

Discussion

A minimum of eight antigens have been demonstrated by gel diffusion analysis of the electrophoretic bands of CB-1A and ten subfractions of CB-1A. Qualitatively, the results support the previous observation that that portion of CB-1A precipitated by Ponceau S contains a principal antigen estimated as 85 per cent of the total fraction with lesser antigens comprising the remainder.[9] The advantage of gel diffusion analysis of the antigens in each electrophoretic band is that minor antigens were concentrated sufficiently so that they could be detected.

The results show that each discrete band obtained by cellulose acetate electrophoresis of CB-1A does not indicate a single separated antigen. The number of antigens per band was from one to a maximum of five. Of the 66 bands obtained, only ten gave a single line of precipitate. Seven of these single lines were accompanied by a haze indicative of possible presence of at least one more antigen. Furthermore, these single lines could have been due to a lesser amount of material in the bands than in other bands showing more antigens. Twenty-two bands had at least two antigens, 18 bands had at least three antigens, 15 bands had at least four antigens, and one had at least five antigens.

It is noteworthy that fraction D4, which amounted to only 0.17 per cent of CB-1A,[7] contained five distinct antigens in band d. From this result it seems possible that further purification of the other fractions might reveal the presence of additional antigens on electrophoresis.

Notwithstanding the five antigens shown in band d of fraction D4, the principal specificity, antigen I, apparently constitutes the major proportion of D4. It has been shown that fraction D4 completely absorbed antibodies specific for all the antigens in CB-1A as well as in all of the separated fractions of CB-1A.[7]

The carbohydrate-free allergenic fraction, CB-65A, is of especial interest because of electrophoretic fractionation is due to differences in the protein components, per se, and cannot be due to differences attributed to the effect of protein combined with polysaccharide. Layton and associates[13] separated CB-65A into five bands by means of paper electrophoresis. All five bands gave positive skin reactions when tested on two castor bean sensitive persons. Layton presented no evidence to show whether each band contained a distinct specificity. But, in discussion of paper electrophoresis of CB-1A, he interpreted separate bands as indicative of separate specificities.[11] From the gel diffusion analysis of the separated bands obtained on cellulose acetate electrophoresis of CB-65A, Figure 2, it is apparent that the principal antigen I, predomi-

nates in bands a, b, c, d, and e with a trace in band f. Likewise antigen II is present in bands f, e, d, c, and b with a trace in band a. A third antigen, probably III, is present in bands e, d, c, and b.

Fraction CB-65A was resolved into four bands by electrophoresis on polyacrylamide gel and gel diffusion analysis of the bands showed that all bands contained a common antigen.[8] One other line of precipitate was obtained from band 1.

Specificity relationships of the antigens of CB-65A with those of CB-1A and fractions of CB-1A are shown in Figures 4, 7, and 8, reference 7. The identity of the principal antigen I in both CB-65A and in fraction E1 are shown in Figure 8,[7] where the principal lines of precipitate of CB-65A and fraction E1 join in a reaction of identity.

Layton and associates[11] have stated that our isolation procedure for CB-65A was based on "the premise that the castor seed allergen was a basic protein that was combined ionically in various proportions with polysaccharidic carbohydrate." We have never regarded the linkage of polysaccharide and protein in the CB-1A complex as ionic. Experimental results do not indicate an ionic linkage. We have referred to the polysaccharide as "chemically combined" with protein and consider the linkage as glycosidic.

Band a of most of the fractions contains Antigen I in nearly immunologically homogeneous state. Except for a trace of a second antigen in all bands, the Ponceau S complex of fraction E1 is immunologically homogeneous and it is electrophoretically homogeneous, Figure 1, in that no banding occurred. Fraction E1 is the largest of the fractions described in Table I, reference 7. Antigen I appears to be both immunologically and electrophoretically homogenous in fraction a1, paper XVIII[9] and almost equally pure in fraction b1b and slightly less pure in fraction c2b.[9]

Antigen II appears to be both immunologically and electrophoretically homogeneous in band f of fraction 1A as it occurs as a single line in both Figures 1 and 2. However, as pointed out before, single bands sometimes split up on re-electrophoresis.[9]

There are several possible explanations as to why distinct antigens in the complex mixture, CB-1A, do not migrate as chemically distinct compounds: (a) antigenic proteins may contain a common, identical antigenic determinant grouping yet differ enough chemically to migrate at different rates on electrophoresis, (b) the antigens may be chemically homogeneous but migrate at different rates because combination with other antigens may change their mobilities, (c) association of the same antigens may change the mobilities of the complex, (d) the combination of varying amounts of polysaccharidic carbohydrate chemically combined with the same antigen may affect mobility and (e) the combination of varying amounts of polysaccharide chemically combined with chemically different proteins having the same antigenic determinant groupings may account for differences in mobilities of the antigens. Postulates b and c might account for the electrophoretic migration of a mixture as a discrete band which on re-electrophoresis would separate into more bands owing to a redistribution and regrouping of the components. Whether one or more than one of the foregoing or some other explanation accounts for the observed behavior of CB-1A must await further experimentation.

The data in papers XVI-XIX of this series should be helpful in the isolation, identification and characterization of the other, lesser antigens of CB-1A. As

previously pointed out,[9] both fractions a1 (I) and f2 (II) gave strongly positive skin reactions on a castor bean sensitive person. Fraction a1 appeared to be the most potent. Obviously, one, some, or all of the other observed antigens or even additional antigens of CB-1A also could be allergenic. Further elucidation of the interrelationships of the antigens and allergens of CB-1A must await further experimentation.

Summary

Eight antigens have been demonstrated by gel diffusion analysis of the electrophoretic bands of CB-1A and its subfractions. Highly purified preparations of the principal antigen (I) and a minor antigen (II) have been described. Explanation of the observations that chemically distinct components of CB-1A possessing different mobilities have the same antigenic specificity are discussed.

References

1. Spies, J. R., and Coulson, E. J.: The Chemistry of Allergens. VIII. Isolation and Properties of an Active Protein-polysaccharidic Fraction, CB-1A, from Castor Beans. J Am Chem Soc 65:1720, 1943.
2. Spies, J. R., Coulson, E. J., Chambers, D. C., Bernton, H. S., and Stevens, H.: The Chemistry of Allergens. IX. Isolation and Properties of an Active, Carbohydrate-Free Portein from Castor Beans, J Am Chem Soc 66:748, 1944.
3. Coulson, E. J., Spies, J. R., Jansen, E. F., and Stevens, H.: The Immunochemistry of Allergens. VIII. Precipitin Formation and Passive Transfer Reactions with Allergenic Proteins from Cottonseed and Castor Beans, J Immunol 52:259, 1946.
4. Coulson, E. J., Spies, J. R., Stevens, H., and Shimp, J. H.: The Immunochemistry of Allergens. X. Anaphylactogenic Properties of Allergenic Fractions from Castor Beans, J. Allergy 21:34, 1950.
5. Spies, J. R., Coulson, E. J., Chambers, D. C., Bernton, H. S., Stevens, H., and Shimp, J. H.: The Chemistry of Allergens. XI. Properties and Composition of Natural Proteoses Isolated from Oilseeds and Nuts by the CS-1A Procedure, J Am Chem Soc 73:3995, 1951.
6. Coulson, E. J., Spies, J. R., and Stevens, H.: The Allergen Content of Castor Beans and Castor Pomace, J. Am Oil Chem Soc 37:657, 1960.
7. Spies, J. R., and Coulson, E. J.: The Chemistry of Allergens. XVI. Ion Exchange Fractionation of the Castor Bean Allergen, CB-1A, and Antigenic Specificity Relationships of the Fractions, J Biol Chem 239:1818, 1964.
8. Morris, R. S., Spies, J. R., and Coulson, E. J.: The Chemistry of Allergens. XVII. Disc Electrophoresis and Gel Diffusion of the Carbohydrate-Free Allergenic Protein, CB-65A, from Castor Beans, Arch Biochem and Biophys 110:300, 1965.
9. Spies, J. R., and Barron, J. K.: The Chemistry of Allergens. XVIII. An Analysis of CB-1A from Castor Beans, Ann Allergy 24:499, 1966.
10. Layton, L. L., DeEds, F., and Moss, L. K.: Fractionation of the Allergenic Proteins of Castor Seed, Federation Proc 19:195, 1960.
11. Layton, L. L., Dante, B. T., Moss, L. K., Dye, N. H., and DeEds, F.: Electrophoretic Fractionation of Soluble Antigenic Proteins from the Seed of *Ricinus Communis* (Castor Beans), J Oil Chem Soc 38:405, 1961.
12. Ouchterlony, O.: Diffusion-in-Gel Methods in Immunological Analysis. II. Progress in Allergy VI. 30, S. Karger, New York and Basel, 1962.
13. Layton, L. L., Greene, F. C., DeEds, F., and Green, T. W., assisted by Yamanaka, F.: Electrophoretic Fractionation of a Carbohydrate-Free Allergenic Preparation From the Seed of *Ricinus Communis* (Castorbean). Am J Hyg 75:282, 1961.

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